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Breast cancer progression may be characterized by a switch from hormone-dependent to hormone-independent growth that involves several cellular alterations and is a major problem in the treatment of breast cancer. Expression of a constitutively activated Raf in ER+ MCF-7 human breast cancer cells results in estrogen-independent growth, suggesting that activation of growth factor signaling pathways through Raf may confer a selective advantage for growth of breast cancer cells under estrogen-deprived conditions. In analyzing the mechanisms underlying this, it was discovered that the prolonged growth of these cells in the absence of estrogen also resulted in loss of ER expression. The work presented here has focused on establishing whether this loss of ER expression was reversible, a very important question that has implications for ER-negative tumors, and on starting to determine the mechanisms underlying both the estrogen-independent growth and loss of ER expression. We have determined that constitutive Raf activity does not result in estrogen-independent activation of ER activity, both by examining estrogen-induced genes and by transient transfection assays with an ERE-reporter construct. We have also determined that loss of ER expression is reversible if the Raf kinase activity is abrogated. Furthermore, ER expression only returns in cells that have significantly decreased Raf.

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Douglas E. Cathey 10/1/97

PI - Signature Date

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** These results are as yet unpublished so they are to remain confidential. **

INTRODUCTION

The progression of breast cancer from estrogen-dependent to estrogen-independent growth represents a critical alteration in phenotype since it is most likely an early step in the progression of breast cancer from estrogen receptor (ER) positive to ER-negative, and ultimately results in resistance to anti-estrogen therapy. Clinically, ~60% of patients are ER-positive at diagnosis, and of these, ~30% do not initially respond to tamoxifen therapy, that is they are resistant to tamoxifen even in the presence of ER. It is conceivable that if the mechanisms underlying this resistance could be reversed, the expression of ER would allow for returned sensitivity to tamoxifen treatment. However, progression to the ER-negative phenotype would seem to preclude the reversal of tamoxifen resistance. ER-negative tumors frequently overexpress growth factor receptors, such as the epidermal growth factor receptor (EGFR) or c-erbB-2 (1-3), as do many ER-negative breast cancer cell lines. Experimentally, the induction of estrogen-independent growth via either selection for growth in the absence of estrogen (4,5) or via the targeted expression of a variety of growth signaling factors (6-9) also frequently results in tamoxifen-resistance. However, in none of these systems has complete loss of ER been observed. Upregulation of these pathways would therefore seem to be an early event in progression to ER-negativity, resulting in an intermediate ER+/estrogen-independent phenotype. Understanding the mechanisms underlying the role of upregulated growth factor signaling in estrogen-independent growth might lead to methods of reversing this in the earlier stages when ER is still expressed.

In order to study these mechanisms, we have established a model of upregulated growth factor signaling in the ER-positive MCF-7 human breast cancer cell line via the stable expression

of a constitutively active Raf kinase (referred to as Δ-raf), an important downstream effector of tyrosine kinase receptor signaling. We have previously reported that Δ-raf expression results in estrogen-independent growth, but the cells remain responsive to estrogen (10). However, while the high Raf activity seems to allow for growth in the absence of estrogen, this high activity is not compatible with growth in the presence of estrogen and continued growth in estrogen-containing medium results in down-regulated expression of the transfected Raf. We were therefore interested in determining the interactions between the Raf-signaling and ER-signaling that results in these behaviors.

We have also previously reported that the long-term estrogen-independent growth of Δ-raf clones results in the loss of ER expression (1996 annual report, manuscript submitted). This loss in expression occurs at the protein and message levels, and is at least partially due to decreased transcription of ER. Some very important questions relating to this inverse relationship between Δ-raf and ER expression arose from these observations. Namely, is the loss of ER expression reversible or was this a permanent transition to the ER-negative phenotype. And second, if ER expression could be returned, would the Δ-raf expression once again be lost. And finally, what are the mechanisms underlying the loss of ER expression mediated by constitutive Raf-1 signaling present in our transfectants. Here we report that re-expression of ER can occur if growth in the presence of estrogen is resumed, and this re-expression occurs concomittantly with the loss of Δ-raf expression that we previously reported to occur in the presence of estrogen. We have also found that although the Δ-raf transfectants display increased basal levels of some normally estrogen-induced genes but not others, transient transfection of Δ-raf into parental MCF-7 cells does not induce expression of an ERE-reporter construct in an estrogen-independent manner.

RESULTS

Loss of ER expression is reversible. Having established that the clones had lost expression of ER, it was next important to determine if this loss was reversible. To first examine this, we took advantage of the fact that ΔRaf expression is down-regulated in our clones during growth in the presence of estrogen or FBS. The clones were, therefore, switched back to growth in FBS and monitored for ER expression by immunohistochemical (IHC) detection using an anti-ER MAB generously provided by Geoff Green. Figure 1 shows the results of this IHC assay with the control transfected pooled cells (HCopool) growing in FBS, and control pool cells long-term adapted to grow in CCS. In both cases, ER expression is evidenced by the positive nuclear staining in these cells, and increased expression depicted by increased staining intensity is observed in cells adapted for growth in CCS. Raf 14c, the clone that is essentially ER negative by Western blot analysis (see 1996 annual report), shows no staining for ER as expected (Figure 2). After 4 passages in FBS (f4), a very small number of cells are beginning to express ER, although the intensity is low. And after 12 passages (f12), more cells express increases ER expression levels, although, cells like these represent a definite minority. In Raf 27c (Figure 3), growth in FBS for 4 passages (f4) also results in re-expression of ER, and by 12 passages (f12), most of the cells are ER + and are beginning to resemble the control pool.

The difference we see between the re-expression of ER in these two clones is very interesting because of a feature of these clones described in the first annual report - that is the loss of Δ-raf

expression in cells growing continuously in FBS. To determine the relationship between re-expression of ER and loss of Δ-raf, we have also stained the same cells for raf. We have previously shown that of all the clones, Raf 14 exhibits the highest Δ-raf levels and the least loss of Δ-raf expression over time in FBS. These features are also observed by IHC when switching Raf 14c back to FBS (Figure 4). While there is a decrease in Δ-raf levels in some cells, all cells do remain positive for Δ-raf. Raf 27c (Figure 5), on the other hand, exhibits a significant loss just after 4 passages with many cells becoming negative, and by 12 passages, there are just a few clusters of positive cells remaining. Thus, the ER re-expression data and the Δ-raf loss of expression suggest that the expression of both proteins is not possible.

To examine this further, we adapted the IHC assay to double- label both ER and Δ-raf in the same cells. Shown in Figure 6 is the double-labeling with Raf 14c, and as observed, virtually all of the Δ-raf + cells are ER negative, and the few ER + cells that do arise have much lower levels of Δ-raf. Now in Raf 27c at this point in growth in FBS (Figure 7), most of the cells are ER + and Δ-raf negative. But if we focus on the areas of Δ-raf + clusters, these cells are ER -, and the ER + cells that are in this field are Δ-raf negative. The last panel represents the very small number of cells that do display positivity for both - but when this happens, the intensity for both is on the lower side. We are currently working out the conditions for a MEK inhibitor to demonstrate that if we block Δ-raf signaling through MEK, ER will be reexpressed in a shorter time-frame than the above tissue-culture time frame allows for.

Δ-raf mediated ligand independent activation of ER does not occur. Ultimately, of course, we are interested in the mechanisms underlying the role of Δ-raf on both estrogen-independent growth (see first annual report) and on loss of ER expression. Given that it has been demonstrated

that growth factor signaling is capable of activating ER in the absence of estrogen, the first obvious question was whether this was happening in our Δ-raf transfectants. We have assessed this in two ways. First, we examined the expression of estrogen-regulated genes in our clones in the absence and presence of estrogen. If ligand-independent activation were occurring, one would expect higher basal levels in the absence of estrogen. And, this is what we observed when northern blot analysis for pS2 was performed (Figure 8A). Except for Raf14c which behaves more like an ER-negative cell line in terms of its pS2 expression, all of the other clones display increased basal levels in the absence of estrogen. However, when we examined another estrogen target gene, progesterone receptor (PR), this time by RNase protection assay, there is no basal expression of PR in either the control cells or the clones (Figure 8B). In addition, unlike the control cells in which PR expression is induced by estrogen, no induction occurs in the clones. Thus we have different results with two different genes. An interesting difference between the regulation of these two genes that may be involved in this differential control lies in that the pS2 promoter contains binding sites for and can be regulated by basal transcription factors such as AP-1. PR, on the other hand, does not.

In another approach, we have also used transient co-transfection assays of Δ-raf and an ERE-luciferase reporter construct into MCF-7 cells (the parental cell line used for the stable transfection). Not only is there no luciferase activity in the cells co-transfected with Δ-raf in the absence of estrogen (Figure 9, compare ERE to NON, and ERE + Δ-raf to ERE + empty vector), there is actually an inhibition of the estrogen-induced luciferase activity when Δ-raf was present. We have performed two additional such assays to rule out any artifacts. First, we used the next downstream effector, MEK, in a constitutively active form (Δ-mek) in the same type of luciferase transfection assay. As seen in Figure 10, again there was no estrogen-independent activation of luciferase, and

there was an even more significant repression of the estrogen-induced activity. Second, we went upstream of Raf and used a growth factor that signals through Raf, fibroblast growth factor-1 (FGF-1). And, again there is no estrogen-independent activation in cells treated with FGF-1 (Figure 11). Furthermore, the same inhibition of estrogen-induced acitivity is seen. Taken together, all of this data suggests that while there exists a very intimate relationship between growth factor signaling through Raf and estrogen signaling through ER, this does not occur at the level of estrogen-independent activation of ER activity. Since we have determined this, we have moved onto establishing the effect of Δ-raf signaling on the promoter activity of ER as a means of affecting its activity and expression.

Establishment of tet-repressible Δ-raf clones and initial characterization. In order to get around the effects of growing the cells in culture for periods of time in which other changes could be occurring in the cells, we felt it was it was important to obtain cells which would express Δ-raf in a regulatable fashion. We chose to use the tetracycline-repressible system first described by Gossen and Bujard (11). This system relies upon the presence of tet-operator sequences in the promoter that must be bound by a transactivator protein in order to obtain activation of expression. The transactivator protein is inactive in the presence of tet and becomes able to bind the tet-sequences in the absence of tet, hence the name tet-off is used for this system. We had obtained MCF-7 cells already expressing the transactivator protein from Dr. Fran Kern and had constructed ptet-Δraf. Now unlike our first constitutive stable transfection in which the drug selection marker was contained within the same plasmid as Δ-raf, the ptet-Δraf did not contain a drug selection marker. This required the co-transfection of a pCMV-zeocin as the drug selection marker, and at about the time we were ready to start these experiments, it was beginning to be reported that this

strategy was encountering problems. Apparently, co-integration of the two plasmids is a common occurence, and when this happens, it is possible that the CMV driving zeocin actually starts driving the expression of the gene of interest resulting in basal expression. Because CMV is such a strong promoter, we thought that one way to get around this problem might be to use a much weaker promoter, such as thymidine kinase (TK). We spent some time in constructing pTK-zeocin, which involved PCR amplification of the zeocin gene from pCMV-zeo and then subcloning into a pTK vector. We then stably transfected two clones of MCF-7 cells containing the transactivator protein (referred to as ML-20-27 and ML-20-21) with ptet-Δraf and either pCMV-zeo or pTK-zeo. Unfortunately, the TK promoter appeared to be too weak of a promoter in MCF-7 cells and we did not obtain any drug resistant colonies with this transfection. We did however, obtain many colonies from both of the CMV-zeo transfections. We selected 50 clones from the ML-20-27 (referred to as 27/raf#) cells and 50 clones from the ML-20-21 (referred to as 21/raf#) cells, expanded these clones, and then cultured them in the absence of tet to assay induction of Δ-raf expression. We again used western blotting to determine the level of Δ-raf expression in each clone in the presence and absence of tet. A representative blot of some of these clones is shown in Figure 12. A great number of each of the 50 clones were positive for Δ-raf expression in the presence of tet. This means we were getting basal expression which is what we were trying to avoid. However, since one of our goals is to determine the effects of different levels of Δ-raf expression (low and high) on estrogen-independent growth and apoptosis, we still thought these clones could be useful. But in addition to obtaining low basal levels of Δ-raf, the induced levels of expression (by removal of tet from the media) was quite low, not nearly to the level of Δ-raf expression observed in our original transfectants. Thus, we cannot get both low and high levels of expression from these cells. Since

we had all of these clones however with relatively low levels of expression, we did perform anchorage-dependent growth assays on them to assess their ability to grow in the absence of estrogen. As can be seen in Figure 13, the representative clone shown grows in the absence of estrogen and presence of tet. This means that the low basal levels of Δ-raf being expressed by these cells is sufficient to confer estrogen-independent growth. And as expected, there was not much benefit to removing tet from the media since we do not get significantly higher levels of Δ-raf under these conditions. We have also placed these clones into CCS media and will monitor them for their ability to grow long-term in the absence of estrogen and if levels of Δ-raf increase under such conditions.

Establishment of cells for the tet-on system and flourescent selection. More recently, a tet-activated system has been described. In this case, a four amino acid substitution in the transactivator protein renders it activated by tetracycline instead of repressed, thus the abbreviation of tet-on. The advantage of this method is that tet is added as an inducer and as such works in 24-72 hours, as opposed to the 7-10 days it takes to withdraw tet from cells when it is used as a repressor. We have obtained 13 drug-resistant clones of MCF-7 cells stably transfected with the tet on transactivator (referred to as ML-20 tet-on #), again from Dr. Fran Kern. We have screened these clones for the presence and funtionality of the tet-on transactivator. Because we still want to get around the problems caused by having to cotransfect with CMV-drug resistance gene, we decided to use a newly available bi-directional tet promoter construct from Clontech. In this plasmid, the bi-directional promoter drives expression of the gene of interest in one direction and an enhanced green flourescence protein (EGFP) from the other direction. We are hoping we will be able to use the expression of EGFP as detected by FACS analysis to sort transfected cells expressing Δ-raf instead

of having to use drug resistance. So initially, we have used the pBiTet-EGFP/luciferase plasmid, where luciferase is in place of where Δ-raf would go, in transient transfection assays to screen the 13 clones described above. These results are shown in Figure 14, and from this data, it was established that clones 17 and 15 were capable of the highest induction level of luciferase expression. As seen with clone 15, the tet-induced level is very similar to that obtained with a CMV-luciferase vector. We are now in the process of determining whether we can establish stable transfectants by using the EGFP expression to sort. We have already constructed the pBiTet-EGFP/Δraf vector so that it can be transfected as soon as we work out the conditions for selection.

DISCUSSION

Given the importance of growth factor signaling systems in breast cancer progression and reports on the existence of cross-talk between these signaling systems and the estrogen receptor signaling system, we have been using constitutively active Raf as a model of upregulated growth factor signaling, whether this be by overexpressed EGFR or erbB-2, etc. to study the progression of breast cancer from estrogen-dependent growth to estrogen-independent growth. We have previously found that the expression of a constitutively active form of the Raf kinase in MCF-7 ER+ human breast cancer cells results in two important phenomena with respect to ER activity and expression: it induced estrogen independent growth of these cells, both anchorage-dependent and anchorage-independent, and it resulted in loss of ER expression at the levels of steroid-binding, overall protein, RNA, and transcription. These data were quite exciting because in all other transfections of growth factor signaling components, i.e. EGFR, erbB-2, or Ras, the cells retained ER expression in spite of estrogen-independent growth. Thus, this is the first system described where the chronic activation

of growth signaling pathways has resulted in both estrogen-independent growth and loss of ER. This ER loss could be by one of two mechanisms. First, if Raf activation of the MAPK kinase cascade activates ER in the absence of estrogen (ligand-independent activation), then it is likely that the constitutive activation that occurs in our cells would result in the constitutive activation of ER. And since an end result of ER activation by estrogen is its eventual down-regulation, a constitutive and unending activation of ER would ultimately result in the chronic and total down-regulation of ER. Alternatively, Raf induction of estrogen-independent growth could be via an ER-independent mechanism and the progressive loss of ER in our cells could merely be due to the fact that since the cells no longer need ER for growth, they have down-regulated its expression.

The data presented in this report indicate that estrogen-independent activation of ER activity is not occurring. The expression of estrogen-induced genes in the clones in the absence and presence of estrogen gave conflicting results -- basal levels of one gene, pS2, were higher in the clones than in the control cells, but those of a second gene, PR, were not. However, transient transfection assays with ERE-reporter constructs displayed no such conflict. At different levels of Raf signaling, both upstream with FGF-1 and downstream with Δmek, no estrogen-independent activation was observed. Certainly, evidence for such a relationship between growth factor signaling and activation of ER exists in the uterus, so it was important to determine if the same mechanisms were used in breast cancer cells. Not only does our data indicate that this is not the case, but in addition, we show that growth factor signaling actually represses estrogen-induction, resulting in lower activity than when estrogen is used alone. This has very important implications for the mechanisms behind the Δ-raf induced loss of ER expression. We have preliminary results, obtained by double IHC for ER and Δ-raf, that in the transiently transfected MCF-7 cells, Δ-raf is capable of down-regulating ER

expression in the cells that take up Δ-raf. We are currently confirming this data and establishing whether Δ-mek gives the same results. The interesting feature about this data is that it indicates that the effect on ER expression is quite fast, since the transient transfections are analyzed 48 hours post transfection. The other interesting feature lies in the difference between pS2 expression and PR expression. pS2 has a complicated promoter that can be regulated by several factors including ER and general transcription factors like AP-1. In breast cancer cells, pS2 expression is strictly dependent on expression of ER, suggesting that in these cells, estrogen is the regulator of pS2. Since we don't see estrogen-independent activation in our transient ERE-reporter assays, but we do see high basal levels of pS2 in our clones still expressing ER (except for Raf14c which was close to ER negative at the time of analysis), this raises the question of what is regulating pS2 in these cells. Our first candidate is AP-1, both because of the information mentioned above and because our Raf clones express very high levels of AP-1 activity [a downstream target of Raf signaling(10)]. Furthermore, data is emerging suggesting that AP-1 can regulate ER expression. As a result, we are currently in the process of obtaining ER-promoter constructs to analyze the effect of Δ-raf on these. These effects will then be correlated with AP-1 activity.

We also report here that the ER loss is reversible by down-regulating Δ-raf. Again this is a very exciting and important finding, because it suggests for the first time that the ER-negative phenotype may not be permanent. Clinically, ER-negative patients face a poor prognosis and the use of chemotherapy instead of tamoxifen. Our data suggests that in ER-negative tumors with high growth factor receptor expression and therefore signaling, that abrogation of this signaling may reverse the lack of ER expression. We are currently analyzing breast cancer cell lines with such characteristics (and unmethylated ER gene, see below), to determine if this is the case. We are

determining the conditions for abrogation of Raf signaling in Raf14c cells using a MEK inhibitor and the effect of this on ER expression, and will then apply this to the cell lines just described. However, this presumably would not work for all ER-negative tumors since it has been shown that ~25% of these display methylation of the ER gene thus resulting in its permanent lack of expression. However, our data may have implications for some of the remaining 75% of tumors without ER methylation, and furthermore, the possibility exists that methylation is a late event that occurs only after ER transcription has been repressed for a long time. We are currently addressing this question by monitoring the methylation state of the ER gene in Raf14c cells with continuous ER-negativity. The question is, if we grow these cells under conditions where ER expression is repressed for long enough, will methylation of ER ever occur. This answer would be important because it would suggest that there is a window of opportunity for reversing ER-negativity.

And finally, since the expression of the constitutively active Raf in these cells has such strong effects that the cells are continuously going through adaptive processes and changing (i.e. the loss of Raf expression when the transfectants are grown in FBS in our original report, or the decrease of ER expression, a major focus has been to re-transfect MCF-7 cells with an inducible and regulatable Raf kinase construct. This system, the tetracycline-repressible system, was supposed to allow us to specifically turn on Raf kinase expression and immediately assess the effects of its activity. However as demonstrated in this report, this was not the case due to basal expression of Δraf, 7-10 days to withdraw the tet from the medium, and low induction levels after tet was withdrawn. As described, we are now establishing the tet-on system in such a manner that we should get around several of these problems. First, we are trying to use the enhanced green fluorescence protein on the same plasmid as Δraf as the selection marker to try to get around having to co-transfect a drug

selection gene under the control of a strong promoter like CMV. Second, the tet-on system is off in the absence of tet, and tet is used to turn on expression. The advantage here, is that one does not have to worry about the stability of tet in the media for long periods as in the tet-off system, and furthermore, tet induction is maximal at 24 hrs so that we don't have the long delay of several days as in the tet-off system. In the meantime, since the establishment of the tet-off clones was extremely time and labor intensive, and since they do express low levels of Δraf (regardless of the presence or absence of tet), we are using these clones to determine the effects of much lower levels of Δraf than we had in our original constitutive transfectants on estrogen-independent growth and ER activity and expression. We have so far examined their anchorage-dependent growth in the absence of estrogen in a short-term assay, and have demonstrated that the low levels of Δraf expressed by these cells is enough to confer estrogen-independent growth. We are currently comparing the Raf kinase activity levels between these clones and the original clones, and we are also establishing cultures of these tet-off clones growing long-term in the absence of estrogen.

Material and Methods

Cell Culture. MCF-7 human breast cancer cells were obtained from the Lombardi Tissue Culture Core Facility (originally from Marvin Rich, Michigan Cancer Foundation), and were maintained in Improved Minimal Essential Medium (IMEM, Gibco/BRL,) with phenol red supplemented with 10% fetal bovine serum (FBS, Intergen Company). For growth in the absence of estrogen, media was switched to IMEM supplemented with 10% charcoal stripped calf serum (CCS, Gibco/BRL). For certain experiments, cells were completely stripped of estrogen by repeated rinsing of cells in IMEM and growth in IMEM + 10% CCS (once per day for 4 days) or in others, they were quick-

stripped (3 times per day for 2 days). Hormone treatments, when performed, were with 17β-estradiol (Sigma) at 10^{-8} M or the pure antiestrogen, ICI 182,780 (obtained from Alan Wakeling, Zeneca Pharmaceuticals) at 10^{-7} M. Cells were plated in CoStar (Cambridge, MA) 75 cm^2 T-flasks and grown in a forced air humidified incubator at an atmosphere of 5% CO_2 and 37° C.

Immunohistochemistry Assay. Cells were plated in 2-well chamber-slides (Falcon), allowed to attach, and grow as a monolayer. For ER expression cells were quick-stripped of estrogen. Cells were fixed by incubation for 10 min at rt with 3.7% formaldehyde-PBS, followed by ice-cold acetone for 15 sec. Fixed cells were then blocked by incubation for 60 min at rt in PBS with 1% BSA. For ER detection, cells required permeabilization by incubation in PBS with 0.1% triton X-100 for 5 min at rt. Primary antibody incubations were overnight at rt in a humidified chamber and were at 2.5 μg/ml for anti-ER and at 0.5 μg/ml for anti-Raf diluted in PBS/1%BSA. After 3 PBS washes, secondary antibody incubations were for 60 min at rt and were a 1:200 dilution of biotinylated anti-rat (for ER) and a 1:300 dilution of HRP-linked anti-rabbit (for Δraf) in PBS/1% BSA. Detection of ER required a further incubation of 30 min at rt with streptavidin-alkaline phosphatase (AP) and then visualization with Vector Red (to give a red color). Detection of Δraf required just visualization with DAB (to give a brown color). For double IHC, both primary antibodies were incubated together, followed by both secondaries together, visualization of Δraf, and then streptavidin-AP and visualization of ER. Stained cells were then dehydrated through a graded series of ethanol, followed by xylene, and mounted in cytoseal. All incubations were followed by three washes of 30 sec each and no counterstain was used.

Northern Blot Analysis and RNase Protection Assay. For measurement of expression of progesterone receptor and pS2 mRNA expression in the presence and absence of estrogen, the Δ-raf

clones routinely grown in CCS remained so while the Hcopool control cells in FCS were stripped of estrogen for four days by growth in CCS with daily medium changes. Cells were then treated with or without estrogen for 72 hours. RNA was isolated using RNAzol-B (Tel-Test, Inc.) according to manufacturer's directions.

RNase protection analysis of PR expression was carried out as previously described (8). Briefly, ten micrograms of total RNA was dried and resuspended in hybridization solution containing the appropriate probes. Following RNase digestion, protected fragments were separated on a polyacrylamide gel, dried, and exposed to X-ray film. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was probed as a control for loading.

Northern analysis was carried out on twenty micrograms of total RNA as previously described (8). GAPDH was used to demonstrated equal loading of RNA. The blot was prehybridized in 50% formamide, 5X SSC, 5X Denhardt's, 25 mM NaHPO4 for 4 hours at 42°C. Hybridization was carried out in prehybridization buffer plus 10% (w/v) dextran sulfate overnight at 42°C. The blot was washed twice at room temperature in 0.2X SSC-0.1% SDS and twice in 0.1X SSC-0.1% SDS at 65°C for 20 minutes each. Blots were exposed at -70°C to X-ray film or quantitated with a Molecular Dynamics 445-SI PhosphorImager.

Transient Transfection and Luciferase assays. MCF-7 cells were plated in Falcon 6-well plates, allowed to attach overnight, and were then quick-stripped of estrogens by repeated washing and replacing of the media with IMEM w/o phenol red supplemented with 10% CCS 3 times per day for two days. At the end of the second day, cells were transfected by the calcium phosphate, low CO_2 protocol (12). Briefly, each well was transfected with 2.5 μg of luciferase plasmid and 1.0 μg of of Δraf or Δmek plasmid suspended in $CaCl_2$ and mixed with BBS. The luciferase plasmids were

either pGLB-MERE or pGLB-MNON, obtained by inserting an MMTV promoter containing either a double consensus ERE (MERE) or the same sequence with the ERE palindromes scrambled (MNON) (13) into the Hind III site of Promega's pGLB basic luciferase plasmid. The cells were incubated for 18 hrs at 2% CO_2 and 35° C, were then washed two times with PBS, and then incubated for 48 hrs in media containing vehicle (0.01 % ethanol), 10^{-9} M estradiol, 10^{-7} M ICI 182,780, 10 ng/ml acidic FGF plus 10 ug/ml heparin, or a combination of FGF and estrogen. Cells were assayed for luciferase activity (expressed as relative light units of RLU) using Boehringer Mannheim's kit according to the manufacturers instructions. The luciferase values were normalized for protein to obtain RLU/mg, and the RLU/mg values were adjusted to specific RLU/mg by subtracting out the value obtained with lysate prepared from mock-transfected cells. The duplicates were then averaged, and the values were plotted as specific RLU/mg protein.

Gel Electrophoresis and Western Blotting. Cell lysates were prepared from 75 cm^2 T-flasks in a similar manner as that described previously (10). Briefly, cells were rinsed in PBS and then lysed in the flask in a modified Gold Lysis Buffer [20 mM Tris, pH 7.9, 137 mM NaCl, 5 mM EDTA, 10 % glycerol, 1% Triton X-100, 1 mM EGTA, 1 mM Pef-bloc A (instead of PMSF), 1 mM aprotinin, 1 mM leupeptin, 1 μM pepstatin A, 1 mM bacitracin, 1 mM Na_3VO_4, 1 mM sodium pyrophosphate, 10 mM soduim flouride] on ice, scraped into a microfuge tube, and centrifuged at 12,000 xg to pellet nuclear debris. Supernatants were analyzed for protein content using the BCA protein assay kit (Pierce) and stored at -20° C.

For Raf Western blots, 25 μg of cellular lysate was electrophoresed through 10% SDS-polyacrylamide gels where the separating gel consisted of 10% acrylamide/0.1% bis-acrylamide and the stacking gel was 3% acrylamide/0.25% bis-acrylamide. 0.1% SDS was included in the gel and

running buffers. Rainbow molecular weight markers were from Amersham: myosin, MW 200,000; phosphorylase B, MW 97,400; bovine serum albumin, MW 69,000; ovalbumin, MW 46,000; carbonic anhydrase, MW 30,000; trypsin inhibitor, MW 21,500; and lysozyme, MW 14,300. Electrophoresed gels were transferred to 0.45 μm nitrocellulose (Bio-Blot NC, CoStar Corp.) for 2 hrs at 0.4 amps in Towbin's buffer (20 mM Tris, 150 mM gylcine, pH 8.3, 20% methanol, 0.1% SDS), and the blots were blocked in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) with 5 % BSA and an additional 0.15% Tween-20 added for 60 min at rt. Blots were then incubated with an anti-Raf polyclonal antibody (made against the carboxy-terminal 12 amino acids from the human Raf-1 sequence, Santa Cruz) diluted to 1 μg/ml in TBST plus 1% BSA overnight at 4° C. After washing the blots 3X5 min with TBST, they were incubated with donkey anti-rabbit antiserum linked to horseradish peroxidase diluted 1:4000 in TBST/1% BSA for 60 min at rt. The blots were again washed in TBST, once for 20 min and then 3X5 min, and the bound secondary antibody visualized using enhanced chemiluminescence (ECL, Amersham) according to manufacturer's instructions.

Growth Assays. 21Copool, 27Copool, and ptet-Δraf clones were quick-stripped to remove estrogens from the medium. Cells were trypsinzed and plated in CCS into triplicate wells of 24-well plates. After allowing the cells to plate for 24 hrs, an initial cell count was taken to give the Day 0 counts and various hormone treatments were added to the cells. Cell counts were performed at Day 3 and Day 6 by aspirating the media and incubating the cells in PBS with 10 mM EDTA for 30 min at rt. Cells were counted in a Coulter automated cell counter (Coulter Electronics), the triplicates averaged, and values were plotted logarithmically. Doubling times were calculated from values obtained from the linear portion of the growth curve using the equation :

$$\text{doubling time (hrs)} = t_2 - t_1 / 3.32(\log N_2 - \log N_1)$$

where N_2 is the number of cells at t_2 and N_1 is the number of cells at t_1.

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Figure 1

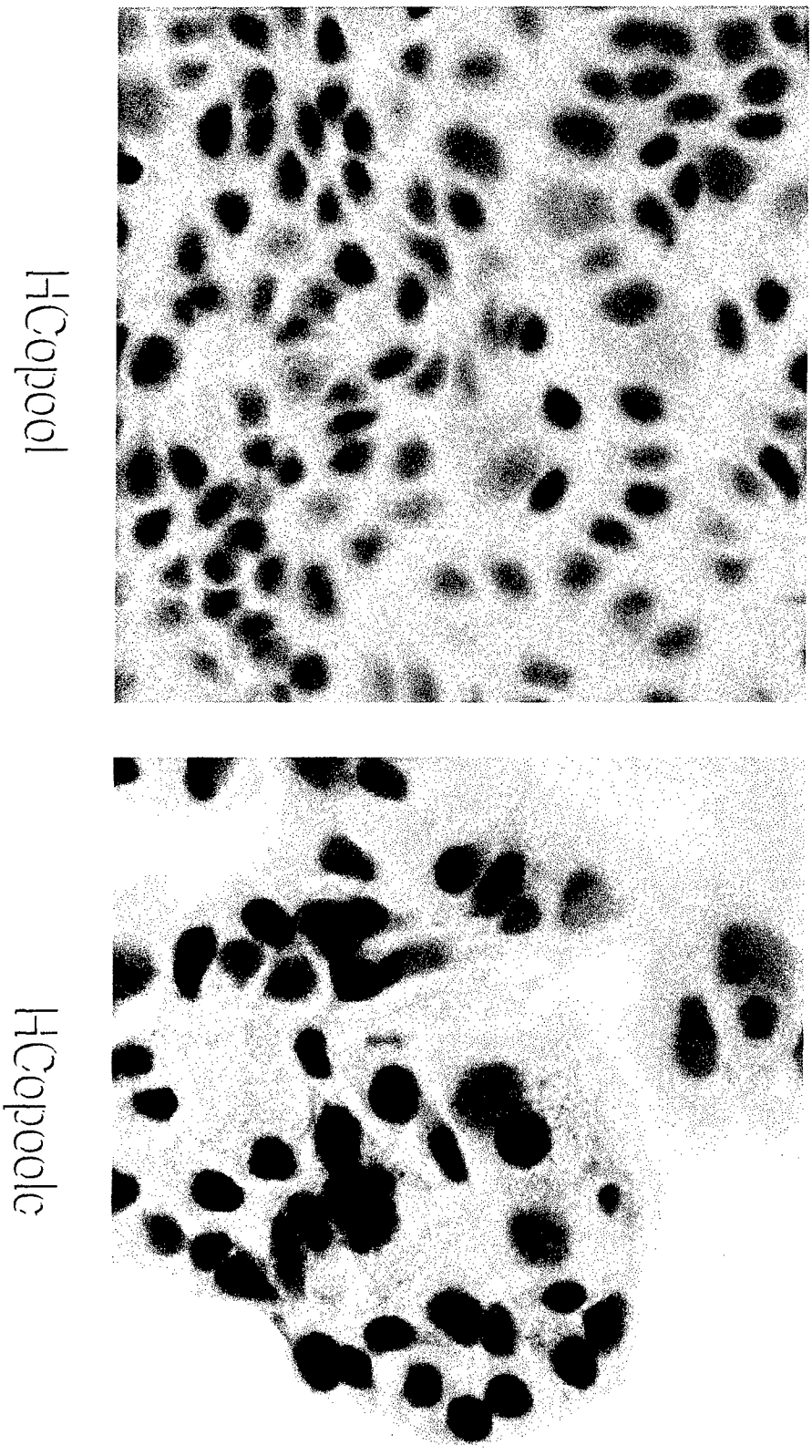


Figure 2

Figure 3

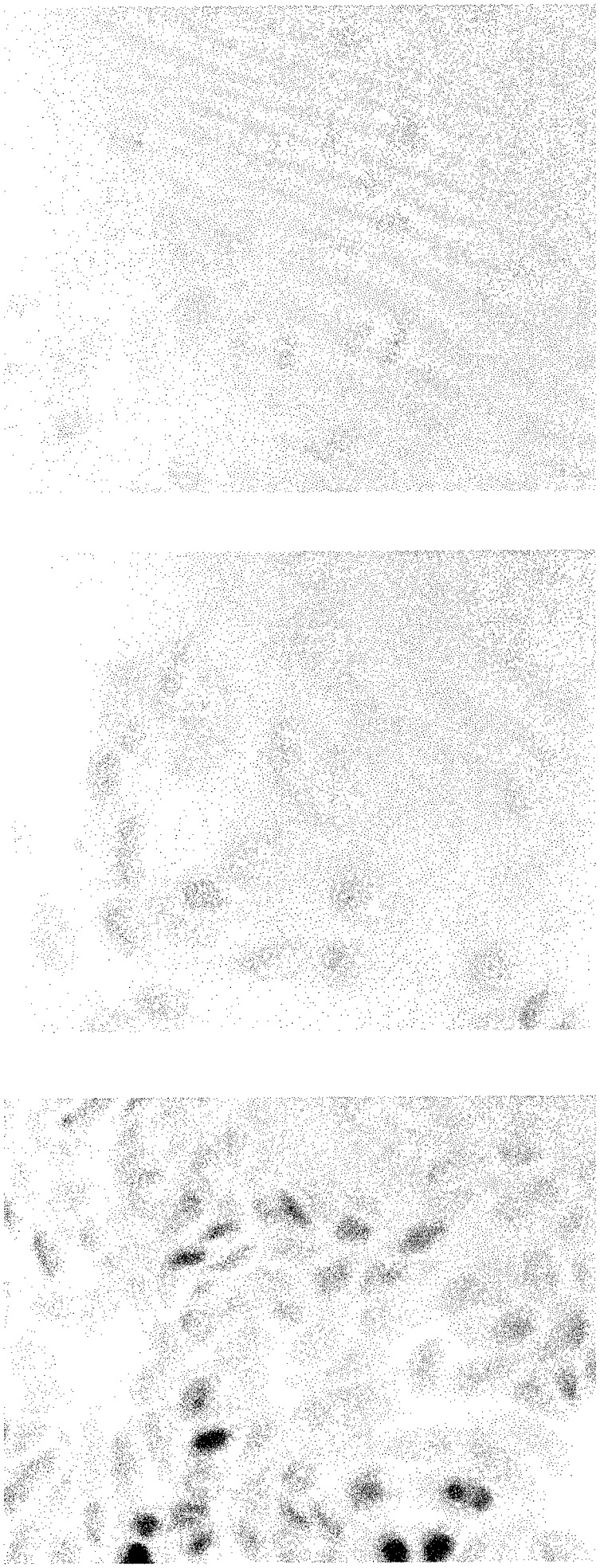
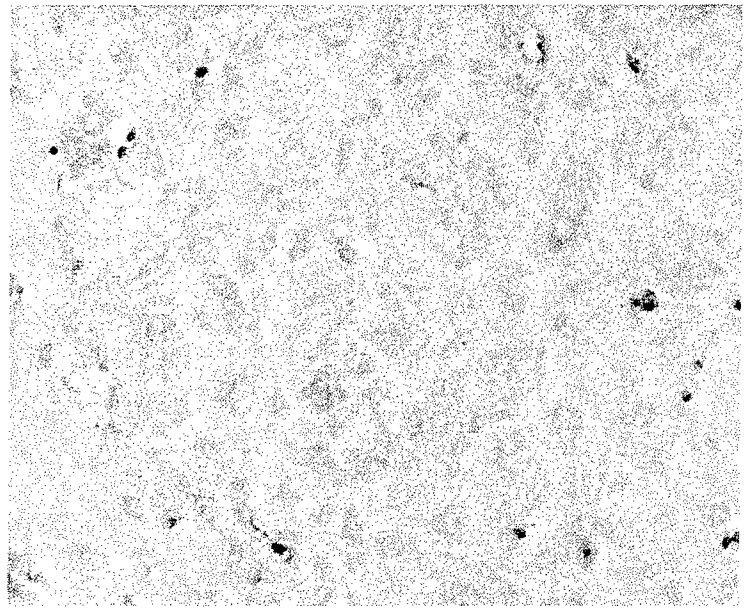


Figure 4

Figure 5



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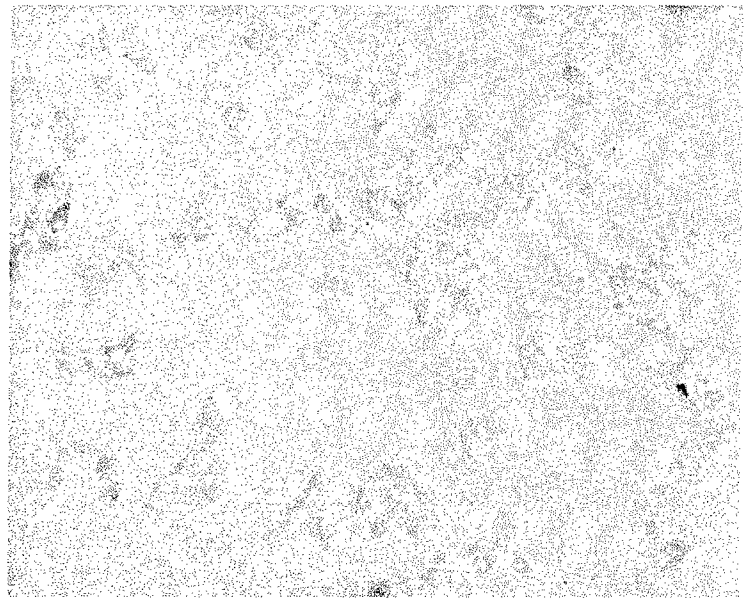

Raf27c-f4

Raf27c-f12

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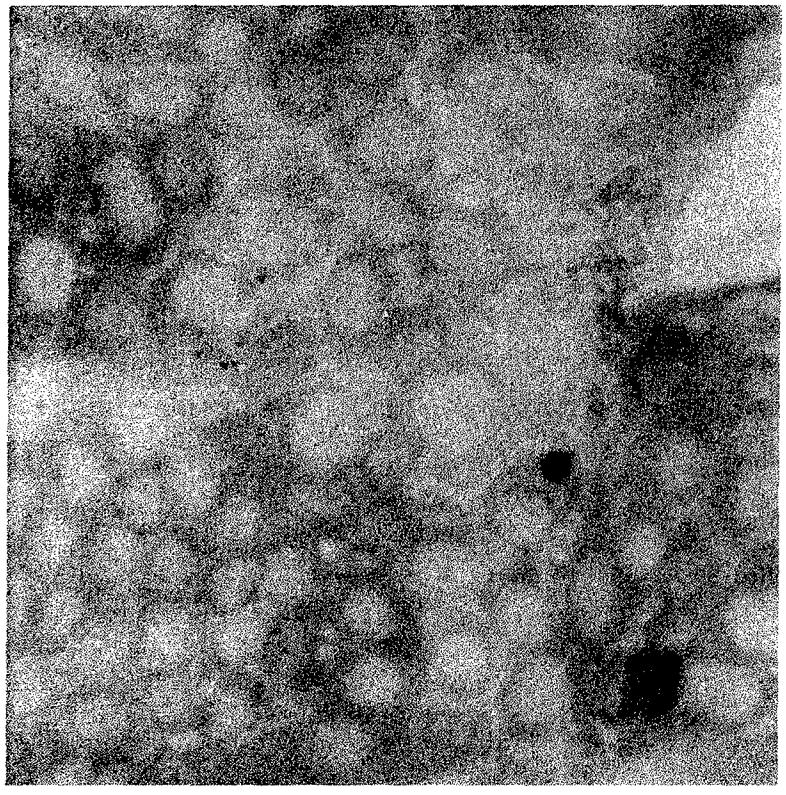

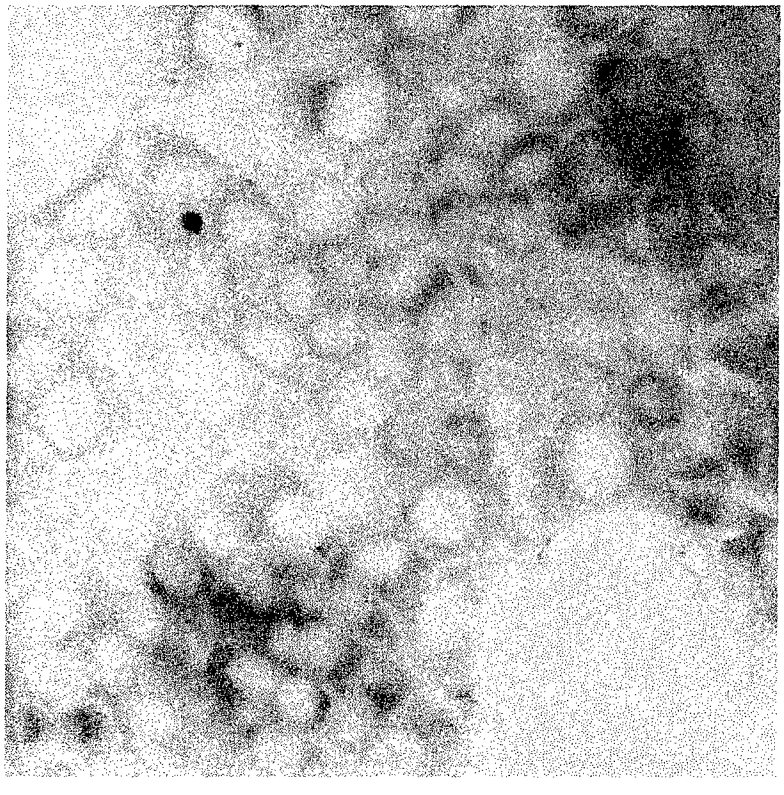

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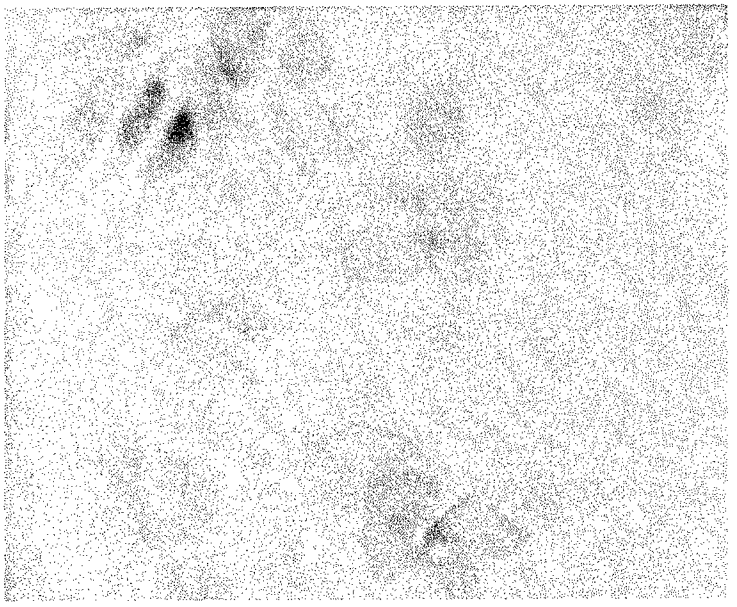

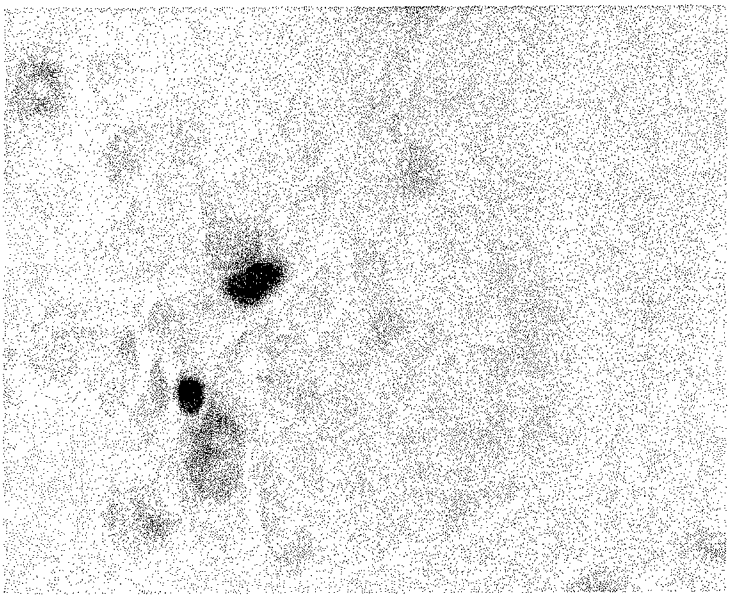

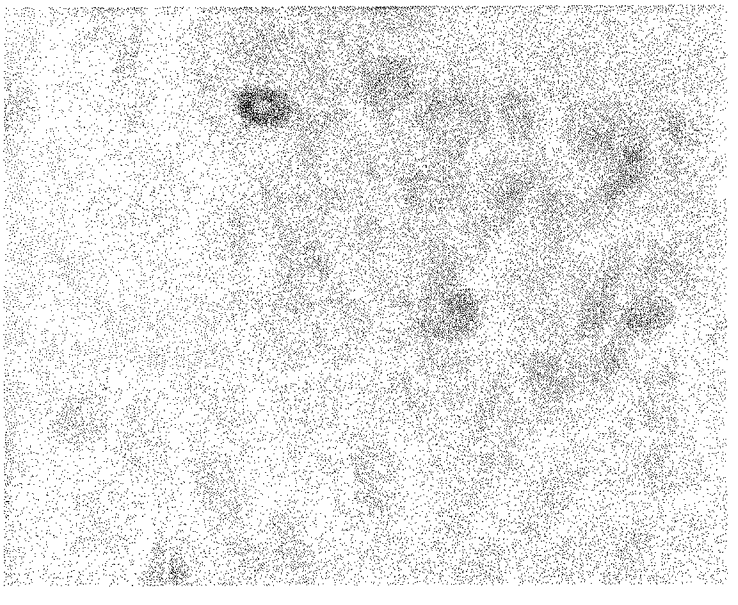

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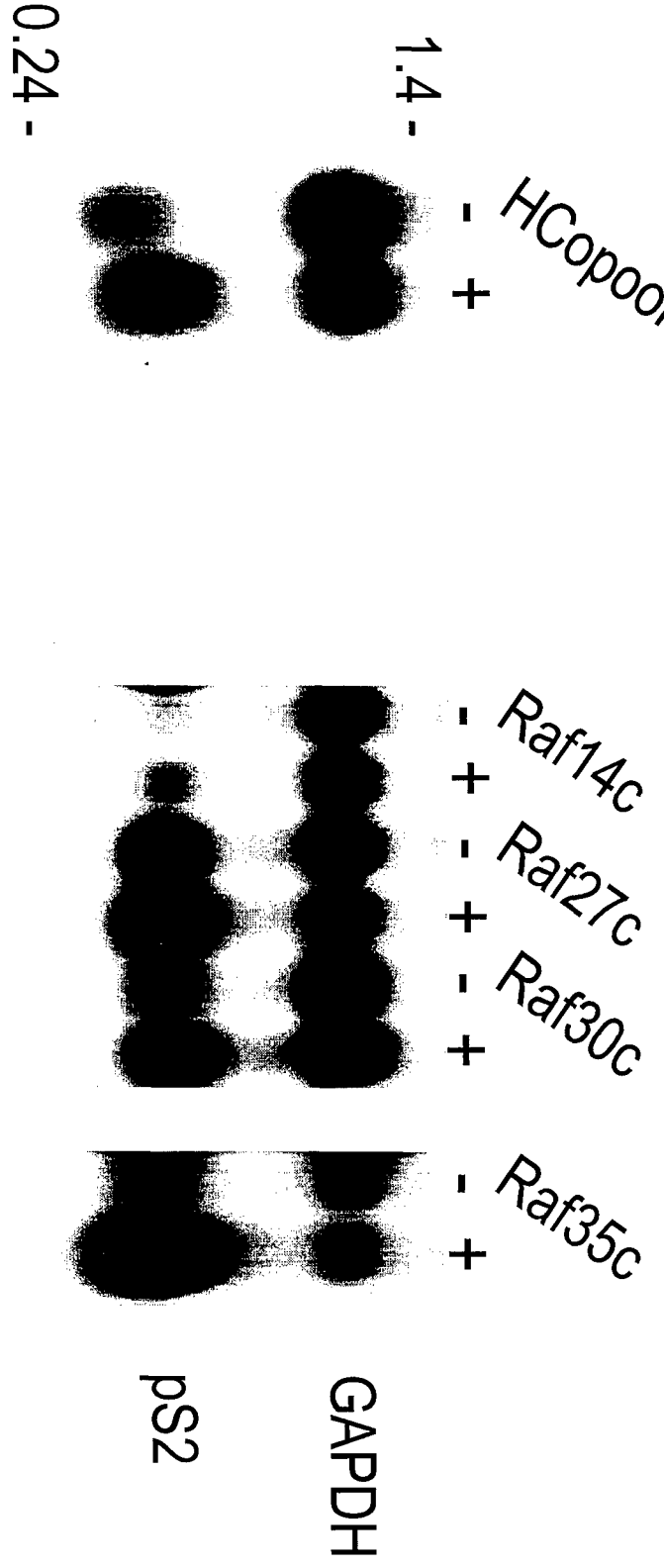

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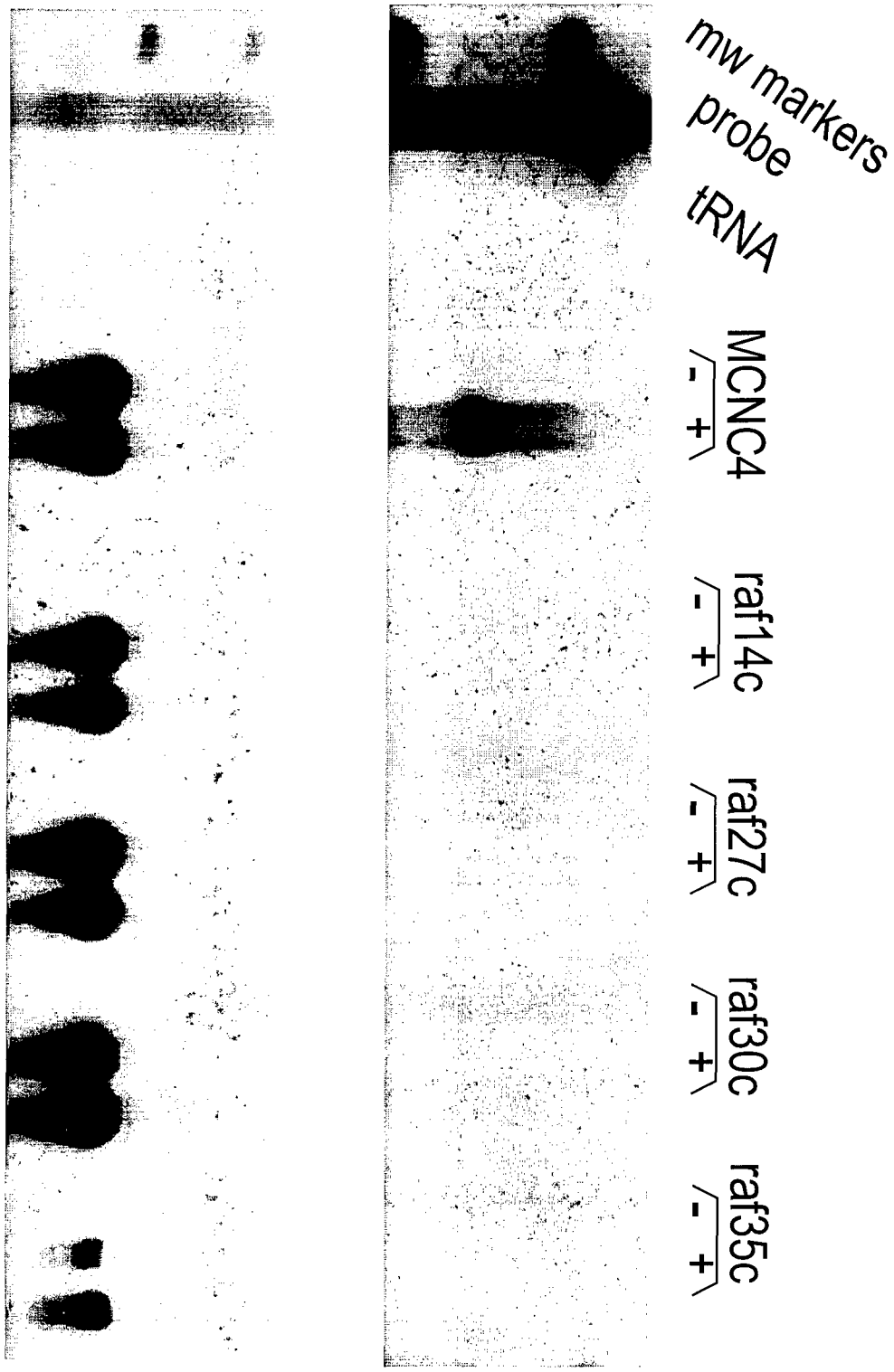

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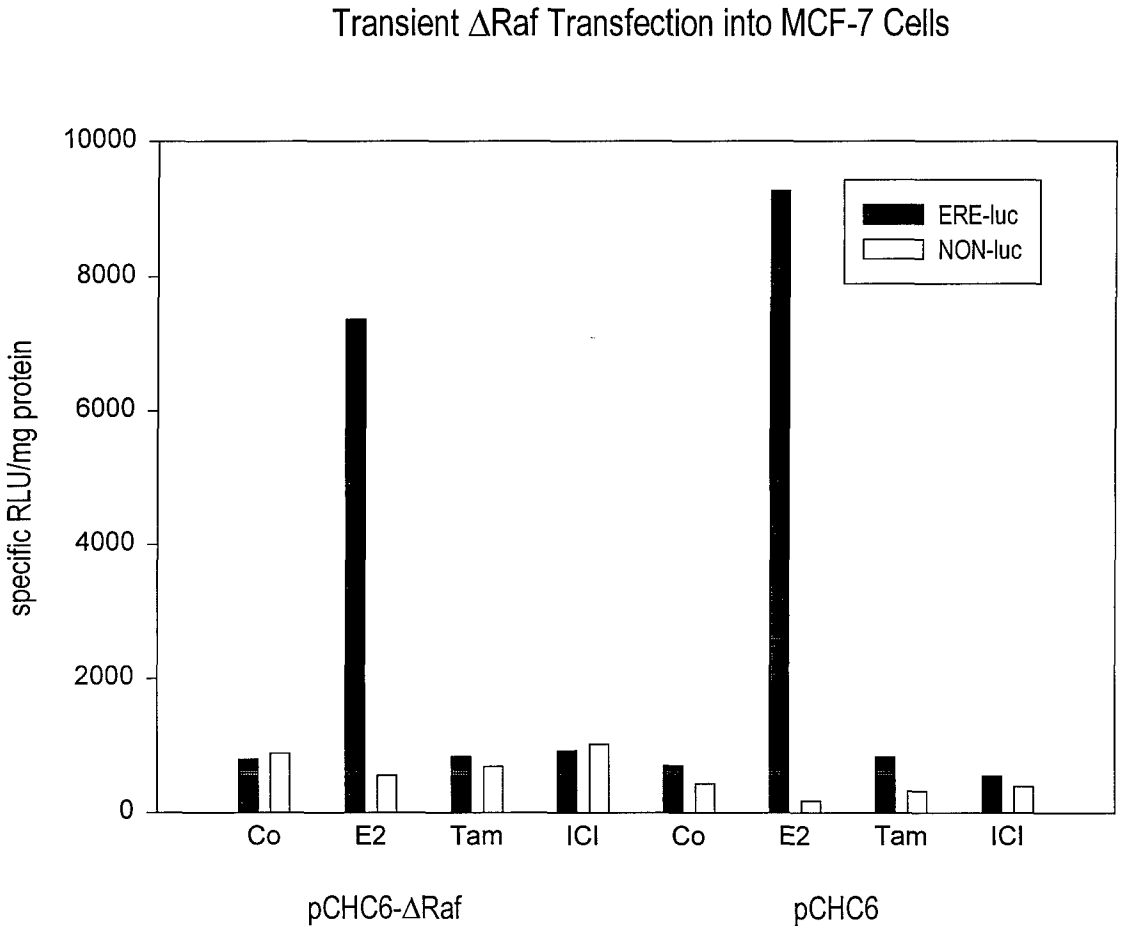

Transient ΔRaf Transfection into MCF-7 Cells
specific RLU/mg protein
10000
8000
6000
4000
2000
0
ERE-luc
NON-luc
Co
E2
Tam
ICI
Co
E2
Tam
ICI
pCHC6-ΔRaf
pCHC6

Figure 10

Estrogen Induction of Luciferase in Transiently Transfected MCF-7 Cells with ΔMEK

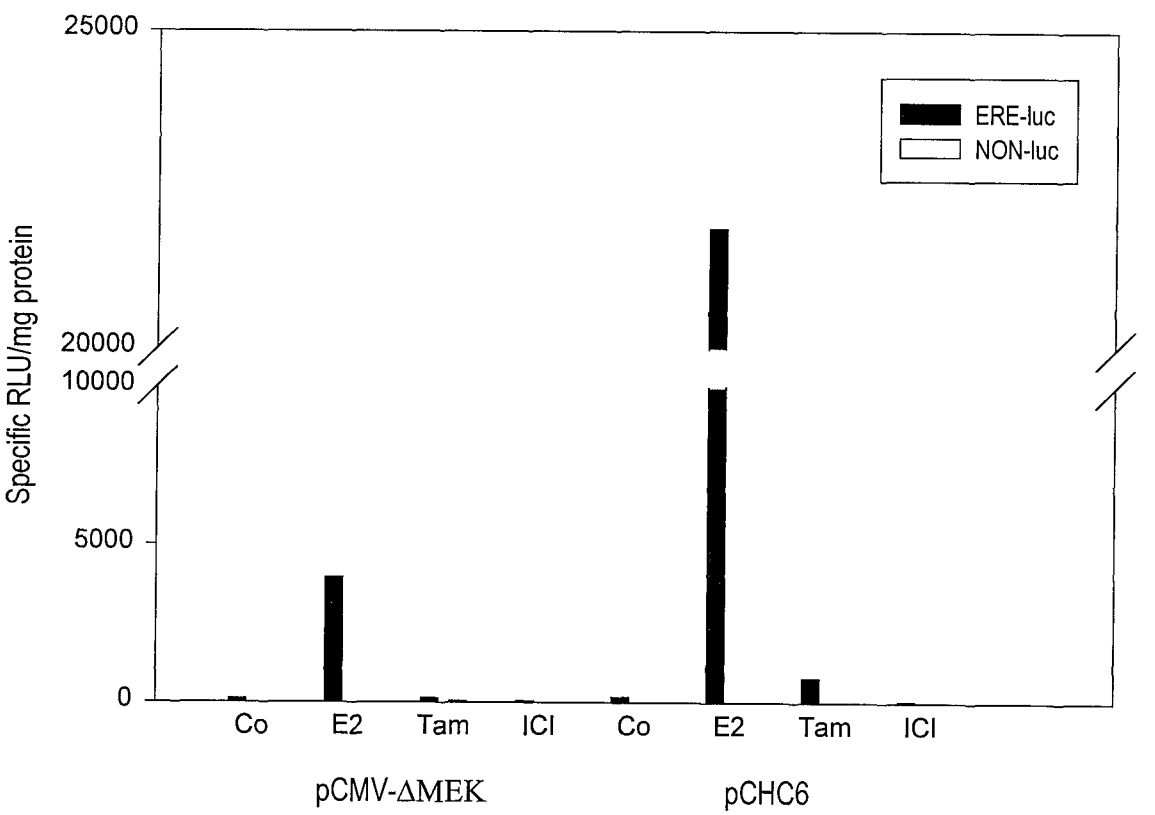


Figure 11

FGF Repression of Estrogen-Induced Luciferase Activity in MCF-7 Cells

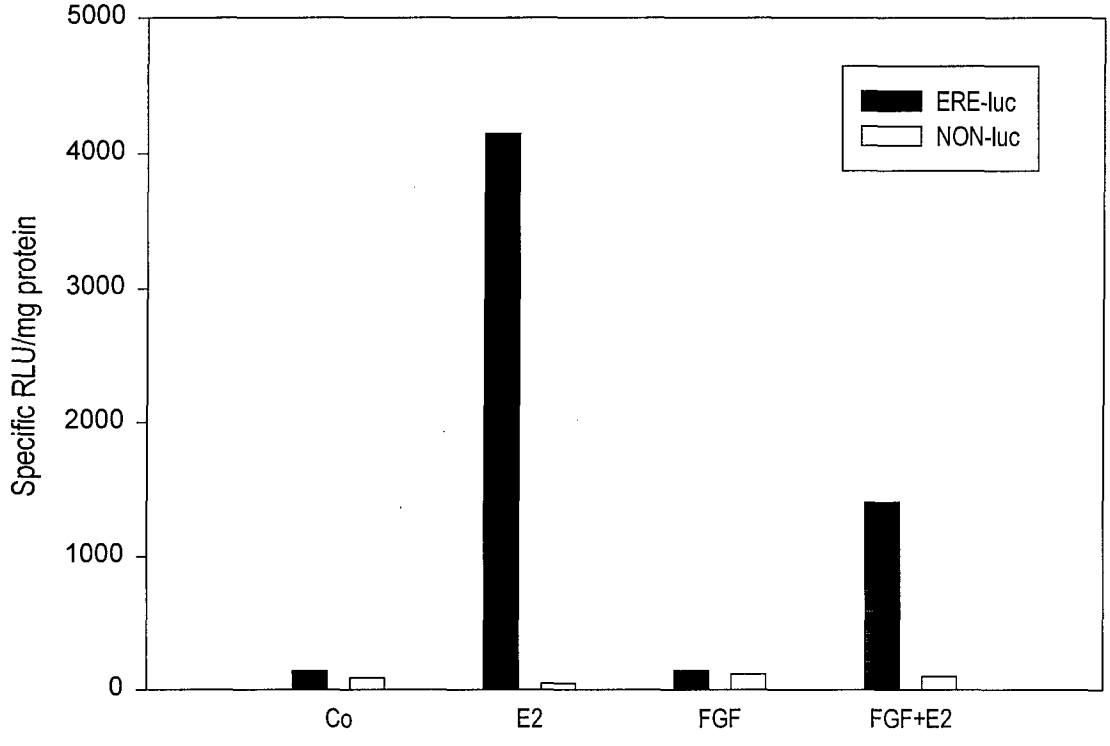
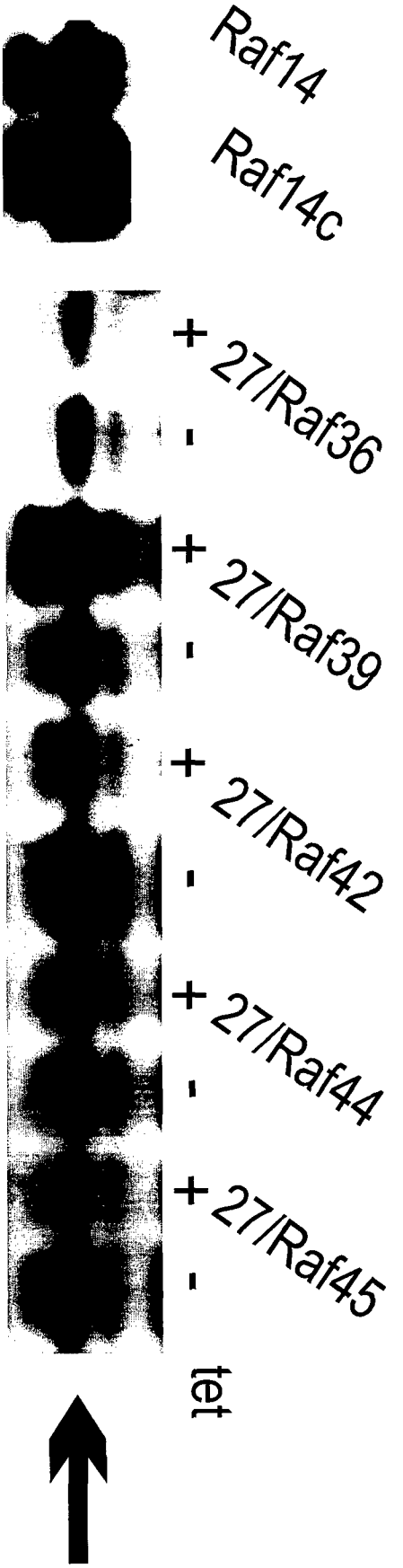
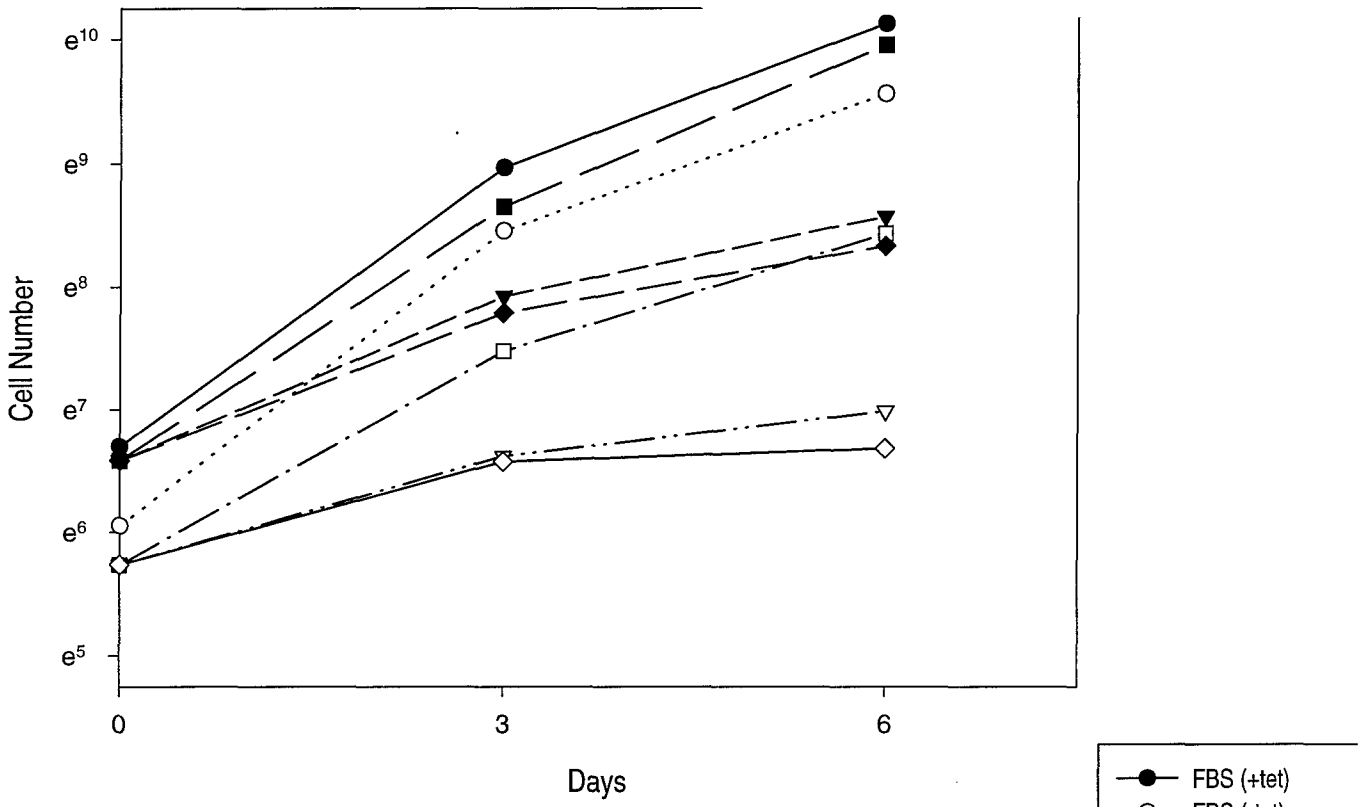


Figure 12



21 Copool

Figure 13



21/21

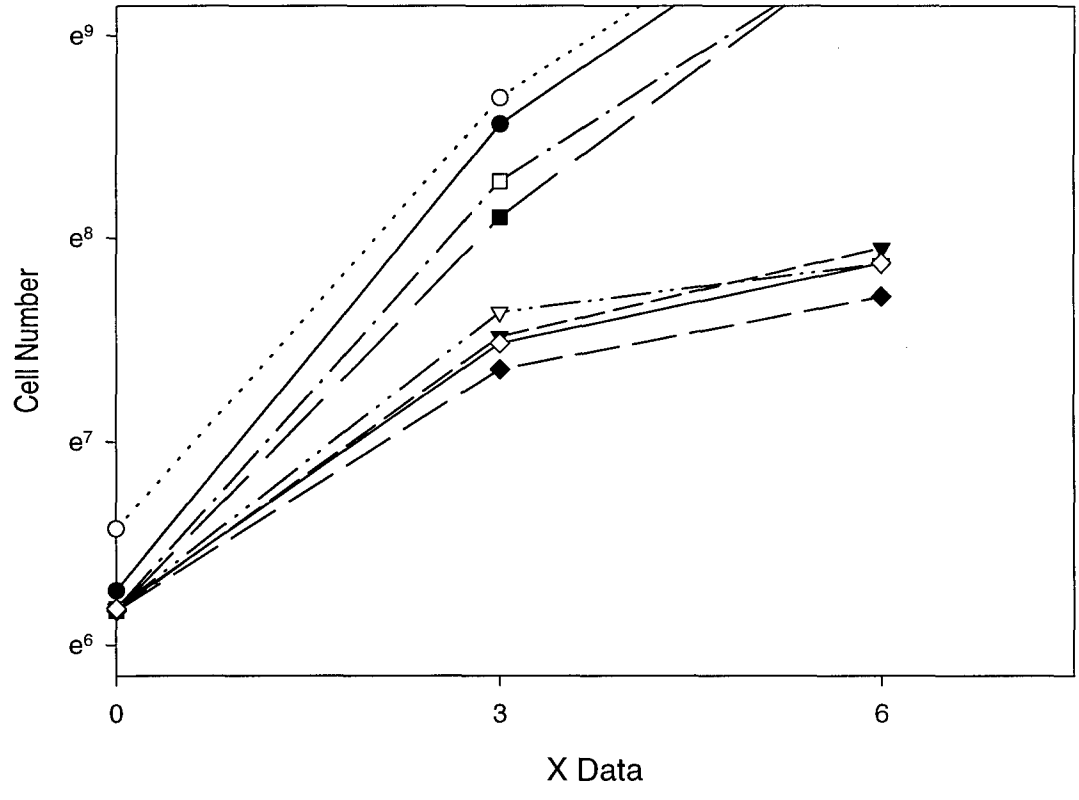
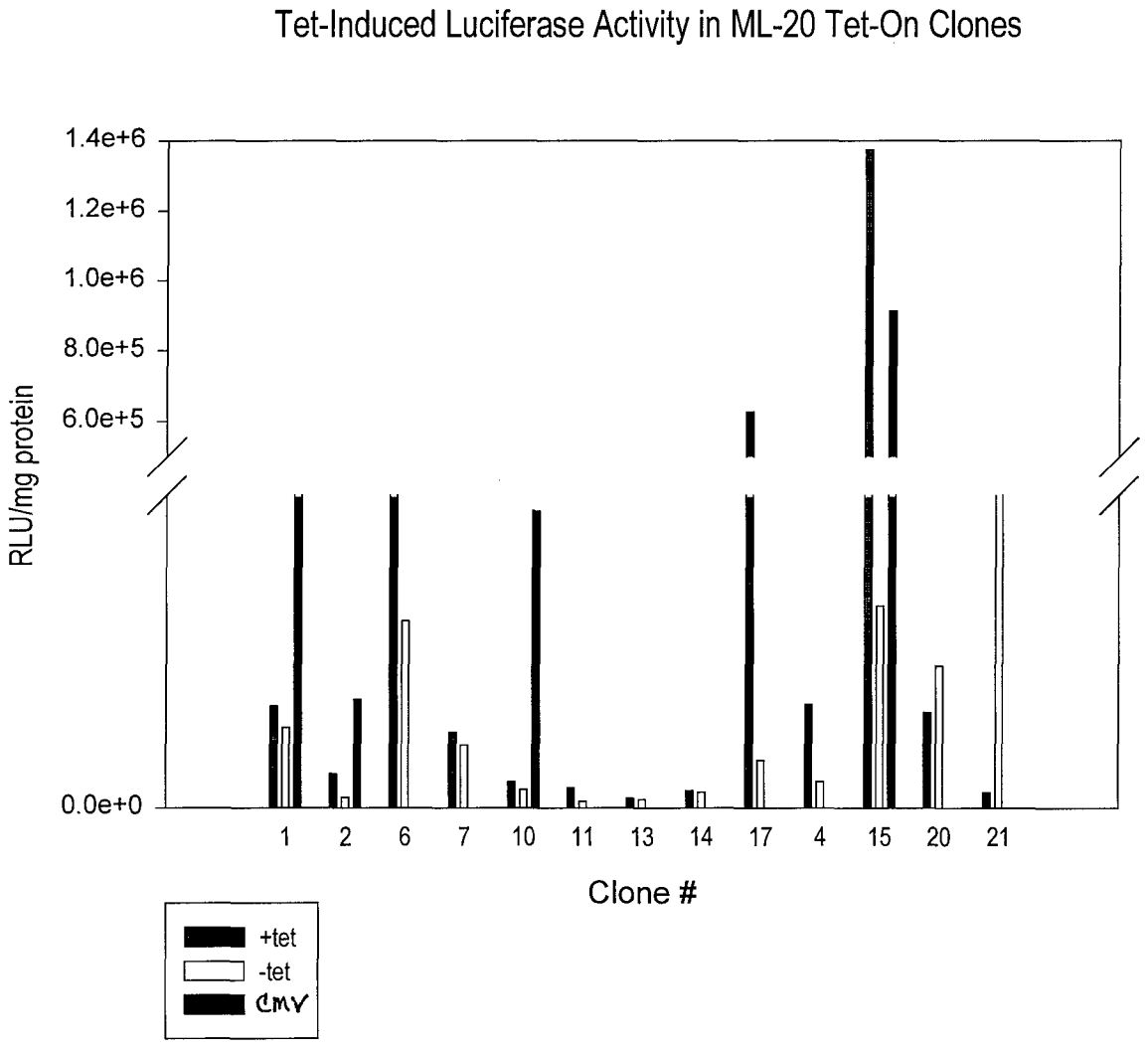


Figure 14


Tet-Induced Luciferase Activity in ML-20 Tet-On Clones
1.4e+6
1.2e+6
1.0e+6
8.0e+5
6.0e+5
0.0e+0
RLU/mg protein
1
2
6
7
10
11
13
14
17
4
15
20
21
Clone #
+tet
-tet
CMV

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 22 Jun 00

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2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at Virginia.Miller@det.amedd.army.mil.

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Information Management